

Attorney Docket No.: RU-0170
Inventors: Lam and del Pozo
Serial No.: 10/009,472
Filing Date: March 29, 2002
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Amendments to the Specification:

Please replace the paragraph beginning on page 8, line 11 with the following rewritten paragraph:

--The inventors have tested this novel strategy for the detection and monitoring of protease activities for *in vitro* and *in vivo* studies. As described in Example 1, the hormone binding domain (HBD) of the rat glucocorticoid receptor (GR) was fused to the bacterial enzyme β -glucuronidase (GUS) with a peptide sequence (YVADG Tyr-Val-Ala-Asp-Gly, SEQ ID NO:21) for caspase-1 cleavage inserted as a linker. When translated *in vitro* with rabbit reticulocyte lysates or expressed in transgenic plants (tobacco and Arabidopsis), the fusion of GUS to the HBD of GR resulted in complete inactivation of its enzymatic activity. *In vitro*, the inventors demonstrated that cleavage of GUS-GR by addition of caspase-1 leads to the release of the GUS protein from the GR domain with concomitant appearance of GUS activity. Introduction of a single point mutation in the P1 position of the caspase cleavage site (YVAAG Tyr-Val-Ala-Ala-Gly, SEQ ID NO:22) abolished cleavage of the fusion protein by added caspase-1 and loss of GUS activation. These results illustrate the principle of the invention by demonstrating that HBD domains of steroid receptors can be used to inactivate enzyme activities of attached protein partners. The fusion junction is accessible to proteolytic enzymes, and cleavage by the appropriate protease leads to the separation of the HBD domain from the enzyme partner and results in the unmasking of the latent enzymatic activity.--

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Please replace the lines 27 and 28 on page 11 with the following rewritten lines:

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--MMP-1      Pro-Gln-Gly-Ile-Ala
              Gly-Gln-Darg-X          PEQGIAGQrX      16--
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Please replace the paragraph beginning on page 17, line 28 with the following rewritten paragraph:

--A prerequisite for the strategy is the accessibility of the protease target site in the fusion protein and the ability of the released enzyme to regain its active state. Prior to the experimental results reported herein, it was entirely unclear whether the cleavage site would be available or, if available, if the released reporter enzyme would be active. We proceeded to test this approach by the strategy outlined in Figure 1. Since Caspase-1 from animal systems have been well characterized, we chose its target sequence YVADG Tyr-Val-Ala-Asp-Gly (SEQ ID NO:21) as our first test case. The coding sequence for the bacterial enzyme β -glucuronidase (GUS) is fused to the HBD of GR with the peptide sequence YVADG Tyr-Val-Ala-Asp-Gly (SEQ ID NO:21) as a linker between the two partners of the chimera. As a negative control for sequence specific cleavage by caspase-1, an almost identical fusion protein between GUS and the HBD is created with the linker YVAAG Tyr-Val-Ala-Ala-Gly (SEQ ID NO:22). The conversion of the aspartate at the P1 position in the linker is predicted to abolish cleavage by caspase-1. If our strategy is operating as designed, we would predict that the two fusion proteins will both be inactive upon their synthesis in the

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presence of HSP90. Upon addition of caspase-1, the cleavage of the ~~YVADG~~ Tyr-Val-Ala-Asp-Gly (SEQ ID NO:21) sequence may lead to the appearance of GUS activity while the fusion with the ~~YVADG~~ Tyr-Val-Ala-Ala-Gly (SEQ ID NO:22) linker should be unaffected.--

Please replace the paragraphs beginning on page 18, line 25 and ending on page 19, line 23 with the following rewritten paragraphs:

--Figure 3 presents the results that demonstrate the linker site between GUS and GR-HBD can be specifically recognized and cleaved by purified caspase-1. The two fusion constructs as well as GUS alone were inserted into a pET vector (Novagen) for T7 RNA polymerase dependent in vitro transcription/translation using a coupled rabbit reticulocyte lysate system (Promega). To visualize the translated proteins, [35S]methionine was incorporated into the newly synthesized products. Figure 3 shows that fusion proteins of about 100 kDa were produced with the two constructs while the GUS alone construct produced a protein with an apparent mass of about 70 kDa. Addition of purified caspase-1 generated cleavage products of 70 kDa and about 30 kDa from the ~~YVADG~~ Tyr-Val-Ala-Asp-Gly (SEQ ID NO:21) containing fusion but not the P1 linker variant. In fact, no obvious proteolysis was detected by caspase-1 was observed with either the P1 linker mutant fusion or the GUS alone control. As expected, the cleavage of the ~~YVADG~~ Tyr-Val-Ala-Asp-Gly (SEQ ID NO:21) containing fusion protein by caspase-1 can be inhibited by addition of the caspase specific peptide inhibitor YVAD-emk.

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To assay for the activity of the fusion proteins, we carried out *in vitro* transcription/translation of the different constructs without radiolabelled methionine. The results are presented in Figure 4. In the absence of caspase-1, either fusion protein show ~~essential~~ essentially no significant GUS activity above the low background present in reticulocyte lysates alone. In a separate experiment, we found that transcription/translation with the GUS alone control can produce high GUS activity with this assay system (data not shown). Thus, the absence of GUS activity with the fusion proteins is likely due to the inactivation of the GUS partner by its fusion to the GR-HBD domain and not due to the presence of inhibitory compounds in the lysate. Upon addition of caspase-1 to the ~~YVADG~~ Tyr-Val-Ala-Asp-Gly (SEQ ID NO:21) containing fusion protein, a dramatic appearance of GUS is observed. This is in contrast to the ~~YVAAG~~ Tyr-Val-Ala-Ala-Gly (SEQ ID NO:22) containing fusion protein, in which case no detectable increase in GUS activity is observed. The unmasking of the GUS activity in the fusion is due to the proteolytic cleavage by the added caspase-1 since inclusion of the caspase specific inhibitor YVAD-cmk abolishes this process. These results demonstrate that the released GUS enzyme partner can function properly as a reporter of active caspase cleavage.--